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Biochemical and metabolic profiles of *Trichoderma* strains isolated from common bean crops in the Brazilian Cerrado, and potential antagonism against *Sclerotinia sclerotiorum*

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ABSTRACT

Some species of *Trichoderma* have successfully been used in the commercial biological control of fungal pathogens, e.g., *Sclerotinia sclerotiorum*, an economically important pathogen of common beans (*Phaseolus vulgaris* L.). The objectives of the present study were (1) to provide molecular characterization of *Trichoderma* strains isolated from the Brazilian Cerrado; (2) to assess the metabolic profile of each strain by means of Biolog FF Microplates; and (3) to evaluate the ability of each strain to antagonize *S. sclerotiorum via* the production of cell wall-degrading enzymes (CWDEs), volatile antibiotics, and dual-culture tests. Among 21 isolates, we identified 42.86 % as *Trichoderma asperellum*, 33.33 % as *Trichoderma harzianum*, 14.29 % as *Trichoderma tomentosum*, 4.76 % as *Trichoderma koningiopsis*, and 4.76 % as *Trichoderma erinaceum*. *Trichoderma asperellum* showed the highest CWDE activity. However, no species secreted a specific group of CWDEs. *Trichoderma asperellum* 364/01, *T. asperellum* 483/02, and *T. asperellum* 356/02 exhibited high and medium specific activities for key enzymes in the mycoparasitic process, but a low capacity for antagonism. We observed no significant correlation between CWDE and antagonism, or between metabolic profile and antagonism. The diversity of *Trichoderma* species, and in particular of *T. harzianum*, was clearly reflected in their metabolic profiles. Our findings indicate that the selection of *Trichoderma* candidates for biological control should be based primarily on the environmental fitness of competitive isolates and the target pathogen.

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Introduction

The genus *Trichoderma* (Ascomycetes, Hypocreales) was first described by Persoon more than 200 y ago, and consists of anamorphic fungi that mainly inhabit soil, organic matter, and decaying trees. *Trichoderma* species are among the most commonly distributed fungi in nature, and are found in ecosystems ranging from tundra to tropical. Their ability to survive in different regions can be attributed to diversified metabolic capabilities and natural competitive aggression.

Trichoderma spp. possess a wide variety of mechanisms for survival and proliferation, including physical attack and degradation of other fungi, and utilization of complex carbohydrates. Based on these characteristics, *Trichoderma* spp. are of considerable economic importance, and are used in commercial applications such as industrial enzyme production, heterologous protein expression, antibiotic production, and biocontrol of plant pathogenic fungi (reviewed by Benítez *et al.* 2004). The potential of *Trichoderma* for biocontrol was first recognized in the early 1930s. Since then, the genus has been extensively investigated as an antagonist of soil-borne plant pathogens, including *Sclerotinia sclerotiorum* (Lib.) De Bary, the causative agent of white mould. This economically important disease affects a wide range of hosts, and constitutes a major problem for centre-pivot irrigated production of common beans (*Phaseolus vulgaris* L.) in the Brazilian Cerrado during winter. The Brazilian Cerrado is a savanna-like region, covering more than 50 % of the country. It is an important agricultural area, with common bean, soybean, and maize as the main crops. The biological control of *S. sclerotiorum* using *Trichoderma* species has proved successful in disease management strategies, by causing a decrease in the incidence of soil-borne fungal pathogens. Such biological control operates by several mechanisms, including competition for nutrients, production of volatile and nonvolatile antibiotics, and production of hydrolytic enzymes (reviewed by Benítez *et al.* 2004). Mycoparasitism appears to be a complex process, involving recognition of plant pathogens by chemotropism, coiling around the pathogen and appresorial formation, followed by production of cell wall-degrading enzymes (CWDEs) and peptaibols, mediated by heterotrimeric G-proteins and mitogen-activated protein (MAP) kinases (Druzhinina *et al.* 2011).

Fungi of the genus *Trichoderma* are prolific producers of hydrolytic enzymes, most of which appear to be chitinolytic (chitinases and *N*-β-acetylglucosaminidases [NAGases]), glucanolytic (β-1,3-glucanases), or proteases (De Marco & Felix 2002; Almeida *et al.* 2007). The cell wall composition of plant pathogens is an important feature for inducing the production of these enzymes. The fungal cell wall is a complex structure, consisting of 80–90 % carbohydrate (mainly polysaccharides such as glucans, chitin, cellulose, and mannans) and 10–20 % proteins, lipids, ions, and organic pigments (Elad *et al.* 1983).

The mechanism of mycoparasitism has been extensively researched. Genomic and proteomic studies have been performed to identify new hydrolytic enzymes (Seidl *et al.* 2009; Monteiro *et al.* 2010); to elucidate the synergism between different hydrolytic enzymes, and also between hydrolytic enzymes and antibiotics (Reithner *et al.* 2005); and to investigate cell signalling during the formation of CWDEs (Silva *et al.* 2009).

Despite the different methodologies employed to explore the diversity of *Trichoderma* species and their potential for biological control, few physiological and molecular studies of CWDEs have been published. The aims of the present study were to characterize CWDE production at the molecular level, and to evaluate the antagonistic abilities and metabolic profiles of different *Trichoderma* strains isolated from the Brazilian Cerrado.

Materials and methods

Microorganisms and culture conditions

Twenty strains of *Trichoderma* spp. and a single strain of *Sclerotinia sclerotiorum* were isolated from different agroecosystems of the Brazilian Cerrado, cropped mainly with common beans (Embrapa Rice and Beans, Santo Antônio de Goiás, Brazil; Table 1). All isolates were obtained from 0 to 10 cm deep, GPS-georeferenced soil samples. The isolates were cultured either on potato dextrose agar (PDA) with 0.1 % Triton 100-X, supplemented with 0.01 % chloramphenicol, or on Komada's medium (Komada 1975), and stored on agar slants at 4 °C prior to use. The *Trichoderma harzianum* (ALL-42) isolate was provided by the Department of Biochemistry and Molecular Biology, Federal University of Goiás, Goiânia, Goiás, Brazil. All microorganisms were recovered on PDA plates at 28 °C prior to use in the experimental procedures.

DNA extraction and rDNA amplification/sequencing

DNA was isolated from frozen and lyophilized mycelium, according to the methodology proposed by Raeder & Broda (1985). The nuclear rRNA gene cluster containing the internal transcribed spacer (ITS) 1 and ITS2, and the 5.8S rRNA gene, was amplified using the primer combination ITS1 and ITS4, following the protocol described by White *et al.* (1990). Similarly, a portion of the translation elongation factor 1 alpha gene (*tef1*) (short fifth intron) was amplified using the primers tef1fw (5′-GTGAGCGTGGTATCACCA-TCG-3′) and tef1rev (5′-GCCATCCTTGGAGACCA-GC-3′), according to the methodology outlined by Kullnig-Gradinger *et al.* (2002). After amplification, the PCR product was subjected to gel electrophoresis in a 1 % agarose gel, and purified using a protocol based on polyethylene glycol (PEG)—20 % PEG, 2.5 M NaCl, 80 % ethanol, and pure ethanol. The purification product was used for sequencing in a reaction using a DYEnamic™ ET Terminator Cycle Sequencing Kit (GE Healthcare, USA), according to the manufacturer's instructions, and electrophoresed using the ABI Prism 3100 (Applied Biosystems).

Analysis of sequence data

DNA sequences were aligned using Clustal X v2.0.12. The terminal regions with poor alignment were manually removed using BioEdit v7.0.5.3, and the remaining portions were aligned together. Sequence analysis of the ITS and *tef1* amplicons was performed using the TrichOKEY 2.0 and TrichoBLAST tools available online at http://www.isth.info/. The sequences were deposited in GenBank (Table 1).

Table 1 – Identification and sequence analysis of the *Trichoderma* strains used in this study.

Isolate	Geolocalization		Identification[a]	Accession number	
				ITS 1 and 2	*tef1*
11/06	−13° 13′ 05.01″	−41° 22′ 36.50″	*T. koningiopsis*	HQ857120	HQ857137
11/11	−13° 13′ 05.01″	−41° 22′ 36.50″	*T. asperellum*	HQ857129	HQ857151
17/06	−16° 29′ 36.59″	−49° 18′ 06.13″	*T. erinaceum*	HQ857130	HQ857133
29/11	−23° 25′ 29.57″	−49° 13′ 28.18″	*T. tomentosum*[a]	HQ857127	HQ857149
356/02	−16° 30′ 12.63″	−49° 17′ 27.13″	*T. asperellum*	HQ857123	HQ857153
357/01	−16° 30′ 07.57″	−49° 17′ 27.01″	*T. asperellum*	HQ857114	HQ857134
360/01	−16° 30′ 10.22″	−49° 17′ 37.12″	*T. asperellum*	HQ857131	HQ857143
362/02	−16° 30′ 09.80″	−49° 17′ 35.05″	*T. asperellum*	HQ857124	HQ857132
364/01	−16° 30′ 06.87″	−49° 17′ 22.10″	*T. asperellum*	HQ857112	HQ857144
364/02	−16° 30′ 06.87″	−49° 17′ 22.10″	*T. tomentosum*[a]	HQ857116	HQ857147
397/01	−18° 56′ 50.55″	−47° 29′ 46.05″	*T. harzianum*[a]	HQ857108	HQ857139
400/01	−18° 56′ 29.60″	−47° 29′ 46.05″	*T. asperellum*	HQ857121	HQ857150
468/02	−15° 39′ 48.08″	−47° 20′ 41.57″	*T. asperellum*	HQ857128	HQ857152
475/02	−15° 39′ 48.08″	−47° 20′ 41.57″	*T. harzianum*	HQ857115	HQ857145
476/01	−15° 39′ 48.08″	−47° 20′ 41.57″	*T. harzianum*	HQ857109	HQ857140
476/02	−15° 39′ 48.08″	−47° 20′ 41.57″	*T. tomentosum*[a]	HQ857119	HQ857146
479/02	−15° 39′ 48.08″	−47° 20′ 41.57″	*T. harzianum*[a]	HQ857118	HQ857142
483/02	−15° 39′ 48.08″	−47° 20′ 41.57″	*T. asperellum*	HQ857110	HQ857154
487/02	−15° 39′ 48.08″	−47° 20′ 41.57″	*T. harzianum*	HQ857113	HQ857138
494/01	−15° 39′ 48.08″	−47° 20′ 41.57″	*T. harzianum*	HQ857125	HQ857155
ALL-42	−17° 46′ 02.25″	−50° 56′ 24.00″	*T. harzianum*[a]	HQ857122	HQ857136

a TrichoBLAST identification using ITS 1–2 and tef1 5th intron and confirmed using Bayesian analysis with *tef1*.

Phylogenetic reconstruction using the Bayesian approach was performed according to Jaklitsch *et al.* (2006), through *tef1*, using Mr Bayes v3.1.2. The best model proposed by Mr Modeltest v2.3 based on AIC was a Hasegawa–Kishino–Yano (HKY) + gamma distribution (G) model. Markov chain Monte Carlo (mcmc) sampling was performed with 1×10^6 generations. The Bayesian posterior probabilities (pp) were obtained from the 50 % majority rule consensus of 15 310 trees (one tree sampled from each of 10 generations), after removal of 4000 first trees using the 'burn-in' process. The tree was rooted with the *tef1* sequence of the OY6607 (FJ619248.1) strain from *Trichoderma longibrachiatum*.

Metabolic profile analysis

The global carbon assimilation profiles were evaluated using the Biolog Phenotype MicroArray technique (Druzhinina *et al.* 2006), with the Biolog FF Microplate. The *Trichoderma* strains were grown in 2 % malt extract agar under ambient laboratory conditions with diffuse daylight at 25 °C. The inocula were prepared after conidial maturation (2–3 d), by rolling a sterile, wetted cotton swab in the area containing the conidia. The conidia were suspended in 16 ml of sterile phytagel (0.25 % Phytagel, 0.03 % Tween 40) in disposable borosilicate tubes (20 mm × 150 mm). The spore solution was mixed manually for 5 s and adjusted to a T_{590} of 75 % ± 3 %. Next, 100 µl of spore solution was transferred to each well of a Biolog FF Microplate. The microplates were kept in the dark at 25 °C. The mycelial growth was assessed by measuring the A_{750} at 12 h, 24 h, and 48 h. Each *Trichoderma* strain was analyzed in three independent experiments, using different inocula. Cluster analysis was performed using Mev v.4.6.1, with Pearson's product–moment correlation coefficient as a similarity statistic, and average linkage clustering as the linkage method.

Volatile metabolites from Trichoderma *spp. against* Sclerotinia sclerotiorum

Separate plates containing PDA medium were inoculated in the centre with a 5-mm diameter mycelial disc containing *S. sclerotiorum* or the different *Trichoderma* strains. The lids were removed, and each plate was inverted and placed on top of another plate. Each plate base was then sealed with a double layer of parafilm. The plates were kept in a biochemical oxygen demand (BOD) incubator at 25 °C with a 12-h photoperiod. The pathogen was grown in the upper plate to avoid interference by spores in the lower plate inoculated with the antagonists. The pathogen colony diameter was estimated when the pathogen completely covered the control plate without *Trichoderma*, and was converted to the percentage of inhibition in relation to the control plate. The experiment was replicated three times for each *Trichoderma* strain. The results were compared using analysis of variance (ANOVA) and means separation by the Scott–Knott test ($\alpha = 5$ %), with SISVAR software. Pearson's coefficient was estimated by correlation analysis, using the STATISITICA 8.0 software and Pearson's correlation.

Dual-culture test

To evaluate the antagonism of *Trichoderma* strains against *Sclerotinia sclerotiorum*, 5-mm diameter discs of PDA medium were taken from the edge of actively growing colonies of fresh fungal cultures, and placed on the surface of a fresh PDA plate

at a spacing of 4 cm. The plates were incubated in a BOD at 25 °C, with a 12-h photoperiod. The evaluation was performed when the pathogen completely covered the control plate without *Trichoderma*.

The evaluation of antagonism was carried out according to the classification proposed by Bell *et al.* (1982): grade 1, *Trichoderma* grows on the pathogen and occupies the entire surface of the medium; grade 1.5, *Trichoderma* grows on 87.5 % of the surface of the medium; grade 2, *Trichoderma* grows on ~66.6 % of the surface of the medium; grade 2.5, *Trichoderma* grows on ~62.5 % of the surface of the medium; grade 3, *Trichoderma* occupies ~50.0 % of the surface of the medium; grade 3.5, *Trichoderma* grows on ~37.5 % of the surface of the medium; grade 4, *Trichoderma* grows on ~33.3 % of the surface of the medium; and grade 5, *Trichoderma* does not grow and the pathogen occupies the entire surface of the medium. The experiment was replicated three times for each *Trichoderma* strain, and the results were compared by ANOVA as described previously.

Enzyme production and enzymatic assays

For hydrolytic enzyme production, 3-mm diameter mycelial discs containing *Trichoderma* strains cultured on MYG solid medium (malt extract, 0.5 %; yeast extract, 0.25 %; glucose, 1 %; and agar, 1.5 %) were inoculated into TLE medium ($CaCl_2 \cdot 2H_2O$, 0.3 g l^{-1}; KH_2PO_4, 2.0 g l^{-1}; $(NH_4)_2SO_4$, 1.4 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.3 g l^{-1}; urea, 0.3 g l^{-1}; peptone, 1.0 g l^{-1}; and 0.1 % trace elements [Fe^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}] solution, 0.1 %) containing lyophilized and macerated cell walls of *Sclerotinia sclerotiorum* (0.5 %) as the carbon and nitrogen source. The cultures were grown in 125-ml conical flasks with constant shaking (120 rpm) at 28 °C. After 48 h, the mycelium was harvested by filtration through filter paper, and the culture filtrate was used as a source of enzymes. The cell wall of *S. sclerotiorum* was obtained by culturing *S. sclerotiorum* in MYG liquid medium at 28 °C with constant shaking (180 rpm). After 15 d, the mycelium was recovered by filtration and washed thoroughly with distilled water. Following inactivation by autoclave, the mycelium was frozen and ground in liquid nitrogen.

The chitinase, NAGase, acid phosphatase, β-glucosidase, α-mannosidase, and α-L-arabinofuranosidase (ABFase) activities were determined using *p*-nitrophenyl-derived substrates: *p*-nitrophenyl-*NN*-diacetylchitobiose (*p*-NPNNDC) (2.5 mM); *p*-nitrophenyl-β-*N*-acetylglucosamine (*p*-NPNAG) (5 mM); *p*-nitrophenyl-phosphate (*p*-NPP) (5 mM); *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPGluc) (5 mM); *p*-nitrophenyl-α-D-mannopyranoside (*p*-NPMan) (5 mM); and *p*-nitrophenyl-α-L-arabinopyranoside (*p*-NPABF) (5 mM) (Sigma–Aldrich, St. Louis, USA). The reactions were carried out in microplate assay format. The assay mixtures contained 10 µl of enzyme solution, 40 µl of *p*-nitrophenyl-derived solution, and 100 µl of 50 mM sodium acetate buffer. The mixtures were buffered at pH 4.8 (acid phosphatase/ABFase), pH 5.0 (chitinase), pH 5.5 (NAGase), and pH 6.0 (β-glucosidase/α-mannosidase). After incubation at 37 °C for 15 min, the reaction was stopped by the addition of 100 µl of Na_2CO_3 (1 M). The amount of *p*-nitrophenol was determined spectrophotometrically at A_{405}. One unit of enzyme activity was defined as the amount of enzyme necessary to release 1 µmol of *p*-nitrophenol per minute.

The lipase activity was determined using *p*-nitrophenyl-palmitate (*p*-NPPa) (Sigma–Aldrich, St. Louis, USA) as a substrate, at a concentration of 5 mM in acetonitrile. The assay mixtures contained 100 µl of enzyme solution, 20 µl of *p*-NPPa solution, and 100 µl of 0.1 mol l^{-1} sodium phosphate buffer (pH 7.0) containing 0.27 M NaCl and 0.9 % v/v Triton X-100. After incubation at 37 °C for 30 min, the mixtures were transferred to a domestic microwave oven and irradiated for 30 s on medium-low power to stop the reaction.

The β-1,3-glucanase activity was determined using laminarin (Sigma–Aldrich, St. Louis, USA) as a substrate, at a concentration of 0.75 % w/v in sodium acetate buffer (50 mM, pH 5.0) (Ramada *et al.* 2010). The assay mixtures contained 10 µl of enzyme solution and 20 µl of laminarin solution. After incubation at 50 °C for 10 min, 100 µl of 3,5-dinitrosalicylic acid (DNS) were added, and the mixtures were reincubated at 95 °C for 5 min. The amount of reducing sugar was determined spectrophotometrically at A_{540}. One unit of enzyme activity was defined as the amount of enzyme necessary to release 1 µmol of reducing sugar per minute.

The proteases activities were determined using azocasein (Sigma–Aldrich, St. Louis, USA) as a substrate, at a concentration of 0.25 % w/v in 50 mM phosphate buffer (pH 5.0), 50 mM citrate buffer (pH 7.0), or 50 mM Tris–HCl (pH 8.5) for acid, neutral, and basic proteases, respectively. The assay mixtures contained 20 µl of enzyme solution, 40 µl of azocasein solution, and 40 µl of the respective buffer. After incubation at 37 °C for 30 min, 100 µl of trichloroacetic acid (TCA) 10 % w/v were added, and the mixtures were reincubated at 4 °C for 10 min. The samples were centrifuged at 2500 rpm for 30 min, after which 100 µl of supernatant were transferred to another microplate containing 100 µl of NaOH (1 M). The amount of protease was determined spectrophotometrically at A_{450}. One unit of enzyme activity was defined as the amount of enzyme necessary to elevate 1 unit of absorbance per minute.

The protein concentrations of the samples were determined using the Bradford assay (Bradford 1976), with bovine serum albumin (BSA; Sigma–Aldrich, St. Louis, USA) as a standard.

All enzyme assays were replicated three times for each sample. The results were compared using ANOVA and means separation by the Scott–Knott test ($\alpha = 5$ %), with SISVAR software. Only enzyme assays with a standard deviation (SD) of <20 % were accepted.

Results

Molecular identification

The isolates were identified at the species level using the molecular tool TrichOKEY 2.0, based on the ITS sequence. When this was not possible, TrichoBLAST was used for species identification, using sequences ITS1 and ITS2, and the *tef1* 5th intron, as indicated in Table 1. The identification was confirmed by Bayesian analysis of the *tef1* cluster (Fig 1). We identified nine (42.86 %) out of the 21 isolates as *Trichoderma asperellum*, seven (33.33 %) as *Trichoderma harzianum*, three (14.29 %) as *Trichoderma tomentosum*, one (4.76 %) as

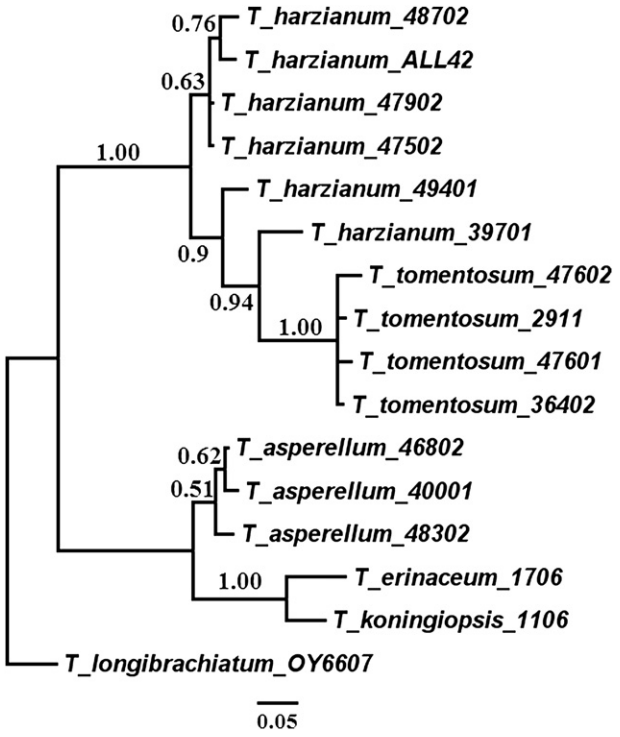

Fig 1 – Consensus tree obtained by Bayesian analysis of the *tef1* sequences with the HKY + G model. *Trichoderma asperellum* strains 11/11, 356/02, 357/01, 360/01, 362/02, and 364/01 are represented by *T. asperellum* 468/02, because they share the same *tef1* sequence.

Trichoderma koningiopsis, and one (4.76 %) as *Trichoderma erinaceum* (Table 1).

Metabolic profile analysis

Separate analyses were performed for mycelial production, by measuring the turbidity (A_{750}), at 12 h, 24 h, and 48 h. Fig 2 shows the turbidity data at 48 h, representing mycelial growth and assimilation of the test substrate. Cluster analysis separated the strains into three major groups (Pearson's coefficient > 0.8): cluster I, a separate group of *Trichoderma asperellum* and one strain of *Trichoderma tomentosum*; cluster II, *Trichoderma erinaceum*, *Trichoderma koningiopsis*, *T. tomentosum*, and *Trichoderma harzianum*; and cluster III, two strains of *T. harzianum*. Additionally, two strains of *T. harzianum* did not group. Cluster I was 70–80 % supported by bootstrap analysis with 1000 iterations, while clusters II and III were 0–50 % supported. Cluster II included two sub-groups, the first containing *T. koningiopsis* 11/06 and *T. erinaceum* 17/06 (80–90 % supported by bootstrap analysis), and the second containing *T. tomentosum* 364/02, *T. harzianum* 476/01, and *T. harzianum* 494/01 (90–100 % supported by bootstrap analysis) (data not shown). The groups containing *T. harzianum* did not correlate with the groups generated by phylogenetic analysis, indicating variability among *T. harzianum* species (as previously described by Druzhinina *et al.* 2010). Furthermore, internal clustering within *T. harzianum* showed four separate groups (Pearson's coefficient > 0.8), indicating a metabolic versatility within the species. Our data demonstrate the importance of metabolic profile analysis in identification of *Trichoderma* strains, and also in elucidation of global species relationships.

The mycelial growth assessment shown in Fig 2 includes the analysis of specific metabolic properties of *Trichoderma* species, as indicated by the most assimilable carbon sources. In general, all species grew best on the following carbon sources: D-glucose, glycerol, D-mannitol, *N*-acetyl-D-glucosamine, i-erythritol, gentiobiose, D-arabitol, D-fructose, dextrin, and xylose. By contrast, poor or no growth was observed on glucuronamide, *N*-acetyl-D-galactosamine, sedoheptulosan, *p*-hydroxyphenyl-acetic acid, L-pyroglutamic acid, L-fucose, rhamnose, and D-psicose. The highest mycelial growth was shown by *T. asperellum* and *T. tomentosum* 476/02, followed by *T. harzianum, Trichoderma gamsii*, *T. tomentosum*, *T. koningiopsis*, and *T. erinaceum*. Specific carbon source assimilation was detected only for *T. asperellum* and *T. tomentosum* 476/02, which was the only one to assimilate D-melezitose.

Potential for antagonistic activity

The antagonistic potential of *Trichoderma* strains was assessed by the inhibition of pathogen growth through the production of volatile metabolites, and also by the dual-culture test. Strains *Trichoderma asperellum* 11/11 and *Trichoderma harzianum* ALL-42 showed the highest inhibition (>50 %) of *Sclerotinia sclerotiorum* growth. Most strains showed moderate inhibition (~30 %), while only three strains—*Trichoderma tomentosum* 364/02, *T. asperellum* 400/01, and *T. tomentosum* 476/02—showed inefficient (<10 %) inhibition (Table 2). In the dual-culture test, strains *T. asperellum* 11/11, *T. harzianum* ALL-42, and *T. tomentosum* 29/11 exhibited the lowest Bell's classification scores (indicating highly efficient antagonism) (Table 2). Most of the remaining strains showed moderately efficient or efficient antagonism against *S. sclerotiorum*, with only three strains—*T. asperellum* 356/02, *T. harzianum* 475/02, and *T. harzianum* 479/02—showing inefficient antagonism (Table 2). We detected no statistical difference in growth inhibition of *S. sclerotiorum* between species (Table 2). Furthermore, we detected a positive correlation between volatile metabolite production and antagonism against *S. sclerotiorum* ($r = 0.55$; $p = 0.0069$) (data not shown). This finding indicates that efficient antagonistic strains are efficient inhibitors of *S. sclerotiorum* growth, through the production of volatile metabolites.

CWDE analysis

In general, in the presence of *Sclerotinia sclerotiorum* cell wall, all of the *Trichoderma* strains secreted a number of different CWDEs, including chitinase, NAGase, proteases, β-1,3-glucanase, lipase, acid phosphatase, α-mannosidase, ABFase, and β-glucosidase (Fig 3). The highest chitinase specific activity was exhibited by *Trichoderma asperellum* 356/02 (1.067 $U \cdot mg^{-1}$, $p < 0.001$), followed by *Trichoderma harzianum* ALL-42 (0.626 $U \cdot mg^{-1}$), *T. harzianum* 475/02 (0.460 $U \cdot mg^{-1}$), *T. harzianum* 487/02 (0.452 $U \cdot mg^{-1}$), and *T. asperellum* 483/02 (0.438 $U \cdot mg^{-1}$). The highest NAGase specific activity was exhibited by *T. asperellum* 468/02 (6.148 $U \cdot mg^{-1}$, $p < 0.001$). The highest acid phosphatase specific activity was exhibited by *T. asperellum* 357/01 (4.017 $U \cdot mg^{-1}$, $p < 0.001$), followed by

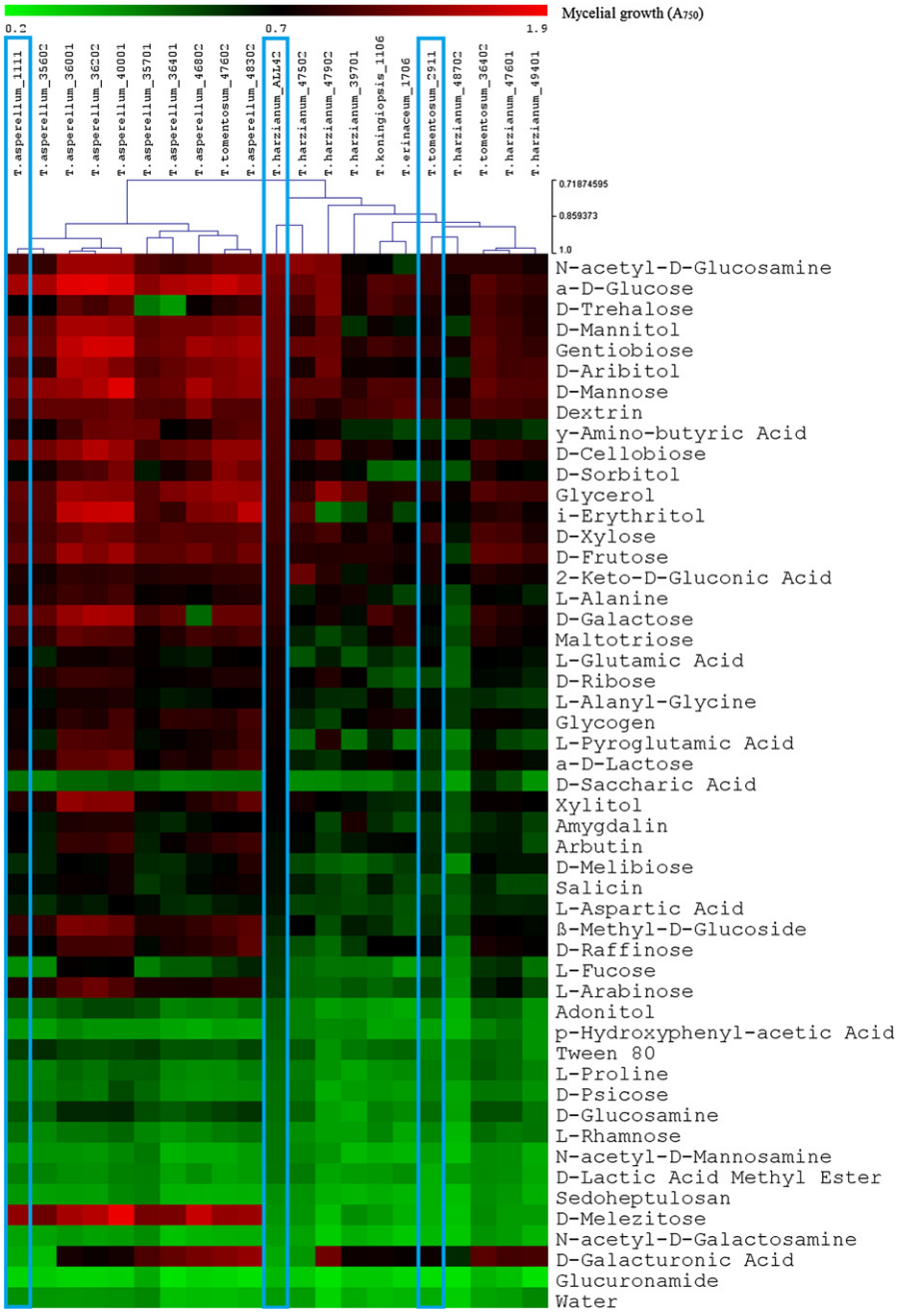

Fig 2 – Global carbon assimilation profiles of *Trichoderma* strains at 48 h. The mycelial growth was assessed by measuring the A_{750}. Low mycelial growth is represented by light green colour ($0.2 \leq A_{750} \leq 0.5$) and high mycelial growth is represented by light red colour ($1.0 \leq A_{750} \leq 1.9$). Intermediate mycelial growth is represented by black colour ($0.5 \leq A_{750} \leq 1.0$). Cluster analyses were based in the grown on fonts of carbon evaluated, with Pearson's product–moment correlation coefficient as a similarity statistic, and average linkage clustering as the linkage method. Each *Trichoderma* strain was analyzed in three independent experiments, with different inocula; data with a mean error of <10 % are shown. Blue rectangles indicate isolates with the highest production of antagonistic volatile metabolites.

T. asperellum 400/01 (3.782 $U \cdot mg^{-1}$, $p < 0.001$), *T. harzianum* 487/02 (2.364 $U \cdot mg^{-1}$), *T. harzianum* 479/02 (1.638 $U \cdot mg^{-1}$), and *T. asperellum* 364/01 (1.457 $U \cdot mg^{-1}$). The highest β-glucosidase specific activity was exhibited by *T. asperellum* 364/01 (1.736 $U \cdot mg^{-1}$, $p < 0.001$), followed by *T. asperellum* 360/01 (1.015 $U \cdot mg^{-1}$, $p < 0.001$), *Trichoderma koningiopsis* 11/06 (0.784 $U \cdot mg^{-1}$), *T. harzianum* 494/01 (0.763 $U \cdot mg^{-1}$), and *Trichoderma erinaceum* 17/06 (0.698 $U \cdot mg^{-1}$). No strain showed exceptionally high specific activity with respect to ABFase, α-mannosidase, or lipase.

The highest β-1,3-glucanase specific activity was exhibited by *T. asperellum* 483/02 (3156 $U \cdot mg^{-1}$, $p < 0.001$), followed by *T. asperellum* 364/01 (3120 $U \cdot mg^{-1}$, $p < 0.001$), *T. asperellum* 468/02 (2703 $U \cdot mg^{-1}$, $p < 0.001$), *T. erinaceum* 17/06 (2669 $U \cdot mg^{-1}$),

Table 2 – Antagonism and growth inhibition of *S. sclerotiorum* by *Trichoderma* strains.

Strains	Species	Antagonism*		Growth inhibition by volatile Metabolites** (%)	
11/06	*T. koningiopsis*	2.00	b	40.67	e
11/11	*T. asperellum*	1.33	a	55.50	e
17/06	*T. erinaceum*	3.00	c	40.00	e
29/11	*T. tomentosum*	1.00	a	31.67	e
356/02	*T. asperellum*	3.33	d	29.33	e
357/01	*T. asperellum*	2.67	c	32.00	e
360/01	*T. asperellum*	1.67	b	33.33	e
362/02	*T. asperellum*	2.00	b	20.00	f
364/01	*T. asperellum*	2.00	b	20.67	f
364/02	*T. tomentosum*	2.67	c	8.33	f
397/01	*T. harzianum*	3.00	c	26.33	f
400/01	*T. asperellum*	3.00	c	8.33	f
468/02	*T. asperellum*	2.00	b	25.00	f
475/02	*T. harzianum*	4.00	d	15.00	f
476/01	*T. harzianum*	3.00	c	24.00	f
476/02	*T. tomentosum*	2.00	b	8.33	f
479/02	*T. harzianum*	3.67	d	23.33	f
483/02	*T. asperellum*	2.67	c	16.67	f
487/02	*T. harzianum*	3.00	c	18.67	f
494/01	*T. harzianum*	2.00	b	21.67	f
ALL-42	*T. harzianum*	1.00	a	60.00	e

*Classification proposed by Bell.
**Similar letters do not differ according to the Scott–Knott test ($\alpha = 5\%$).
a = highly efficient antagonism; b = efficient antagonism; c = moderately efficient antagonism; d = inefficient antagonism; e = efficient growth inhibition; f = inefficient growth inhibition.

T. asperellum 360/01 (2423 $U \cdot mg^{-1}$), and *T. asperellum* 356/02 (2413 $U \cdot mg^{-1}$).

The highest proteolytic specific activity (acid, neutral, basic) was exhibited by *T. asperellum* 364/01 (4.960 $U \cdot mg^{-1}$, 12.770 $U \cdot mg^{-1}$, 18.770 $U \cdot mg^{-1}$), followed by *T. harzianum* 475/02 (4.662 $U \cdot mg^{-1}$, 7.753 $U \cdot mg^{-1}$, 8.077 $U \cdot mg^{-1}$), *T. harzianum* 476/01 (4.414 $U \cdot mg^{-1}$, 7.063 $U \cdot mg^{-1}$, 2.587 $U \cdot mg^{-1}$), *Trichoderma tomentosum* 476/02 (4.335 $U \cdot mg^{-1}$, 7.538 $U \cdot mg^{-1}$, 6.765 $U \cdot mg^{-1}$), *T. harzianum* ALL-42 (3.353 $U \cdot mg^{-1}$, 7.813 $U \cdot mg^{-1}$, 4.406 $U \cdot mg^{-1}$), and *T. asperellum* 483/02 (2.744 $U \cdot mg^{-1}$, 7.143 $U \cdot mg^{-1}$, 8.832 $U \cdot mg^{-1}$). Comparison of the protease profiles revealed no similarity between acid and basic proteases ($p > 0.05$). By contrast, neutral proteases showed a significant similarity with acid, and also with basic, proteases ($p < 0.05$).

Discussion

In this study, we have presented for the first time, a complete molecular and biochemical characterization of *Trichoderma* strains isolated from a Brazilian agroecosystem. We believe that this approach should be used to select potential candidates for commercial biological control against *Sclerotinia sclerotiorum*. Generally, *Trichoderma* strains are identified using morphological and molecular techniques. However, elucidation of the biodiversity of *Trichoderma* has demonstrated that single isolated tools are insufficient to explain genus diversity, particularly within *Trichoderma asperellum* and *Trichoderma harzianum* (Kubicek *et al.* 2003; Hoyos-Carvajal *et al.* 2009). Thus, molecular and biochemical tools have become mandatory for accurate taxonomy. Here, we have identified five different *Trichoderma* species, mainly *T. asperellum* and *T. harzianum*, but also *Trichoderma tomentosum*. Our results are in accordance with those of other recent studies, which have demonstrated a high biodiversity of *Trichoderma* in neotropical regions, with *T. asperellum* and *T. harzianum* as the most frequent species (Kubicek *et al.* 2008; Hoyos-Carvajal *et al.* 2009; Anees *et al.* 2010).

Molecular identification may be difficult, particularly when using only sequences from public data banks such as GenBank, which holds misidentified sequences. In the present study, species identification was supported by a specific database and software, recognized as reliable and advantageous for the identification of new species (Druzhinina *et al.* 2005). Among the 21 strains, only six strains (three isolates of *T. harzianum* and three isolates of *T. tomentosum*) could not be identified using TrichOKEY 2.0. However, these were identified by TrichoBLAST, coupled with phylogenetic analysis (Fig 1). Our data emphasize the need for complementary tools to confirm species identification.

The diversity of *Trichoderma* species, and in particular of *T. harzianum*, was clearly reflected in their metabolic profiles. *Trichoderma harzianum* could be grouped with *Trichoderma erinaceum* and *Trichoderma koningiopsis*, and also with *T. tomentosum*. Furthermore, *T. tomentosum* 476/02 could be grouped with *T. asperellum* (as the only two isolates to metabolize D-melezitose in the Biolog FF Microplate analysis). Hoyos-Carvajal *et al.* (2009) also observed this pattern and suggested the possibility of genetic interchange between *T. harzianum* and *T. tomentosum*. Interestingly, our data suggest a similar metabolic evolution for *T. asperellum* and

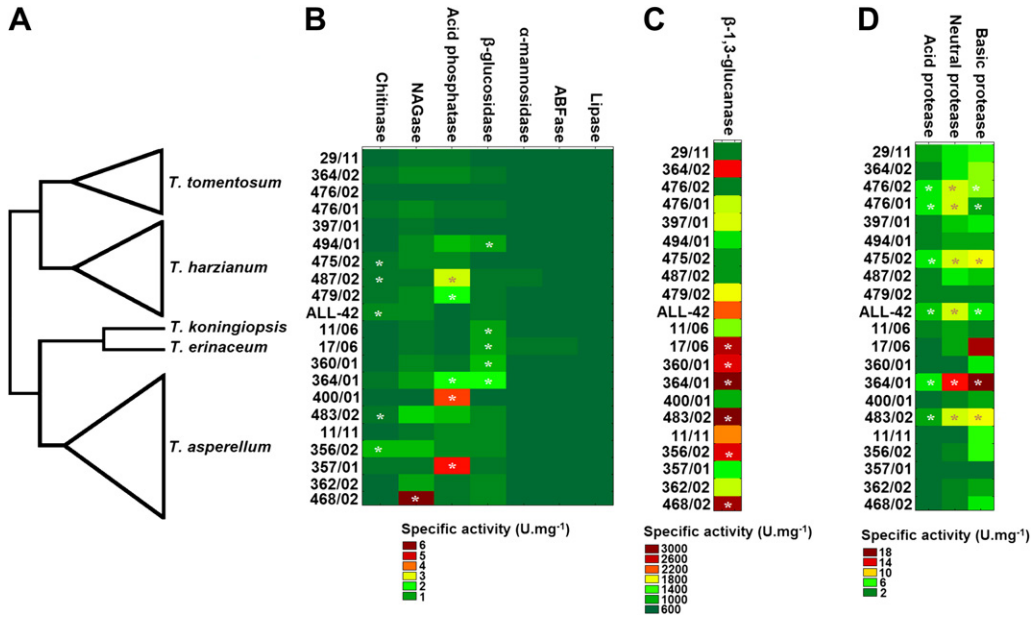

Fig 3 – Specific activities of CWDEs from strains of *Trichoderma* cultured on cell wall extracts of *S. sclerotiorum*. (A) Cladogram obtained by Bayesian analysis of *tef1*. (B) Specific activities of enzymes with *p*-nitrophenyl derivatives as substrates: chitinase, NAGase, acid phosphatase, β-glucosidase, α-mannosidase, ABFase and lipase. (C) Specific activities of β-1,3-glucanase. (D) Specific activities of acid, neutral and basic proteases. All experiments were performed in triplicate; data with a mean error of <20 % are shown. Asterisks indicate the highest specific activities for each enzyme, calculated using ANOVA and the Scott–Knott test (α =Cladogram 5 %).

T. tomentosum. However, genetic interchange between these two species is impossible, because of differences in their genomes (Kubicek *et al.* 2011). Thus, this evolution may have occurred in response to the environment.

Our metabolic data further revealed two distinct clusters for *T. asperellum* (Fig 2). This pattern was also observed by Hoyos-Carvajal *et al.* (2009), indicating a high metabolic diversity in tropical *T. asperellum* isolates. Using a combination of genomic, proteomic, and classical mycological techniques, Samuels *et al.* (2010) demonstrated that *T. asperellum* consists of two cryptic species—*T. asperellum* and *Trichoderma asperelloides* sp. nov. In the present study, we were unable to distinguish these two cryptic species, either by *tef1* or by morphological analysis. Taken together, our findings support the idea of heterogeneity within neotropical *T. harzianum* and *T. asperellum* (Hoyos-Carvajal *et al.* 2009).

The use of metabolic characteristics to investigate phylogeny is increasing. The technique represents an important tool for elucidating species relationships based on ITS sequences. In particular, it may prove valuable in the screening of *Trichoderma* species for industrial applications (reviewed by Atanasova & Druzhinina 2010). In the present study, we have grouped unknown *Trichoderma* clusters (29/11, 364/02, and 476/02) and (397/01, 479/02, and ALL-42) based on ITS and *tef1* sequences, and also on phylogenetic information, into a *T. tomentosum* and a *T. harzianum* cluster, respectively. Moreover, we have identified potential *T. asperellum* candidates with the ability to produce α-1,4 and β-1,4 hydrolytic enzymes, for further investigation in biomass degradation.

The key parameters for success when using *Trichoderma* as a biological control agent are the production of CWDEs, antibiosis, and competition for nutrients (Almeida *et al.* 2007). Mycoparasitism studies have generally focused on the production of chitinases, β-1,3-glucanases, and proteases (De Marco & Felix 2002; Almeida *et al.* 2007; Gajera & Vakharia 2010), all of which are closely related to the cell wall composition of the pathogen (Viterbo *et al.* 2002). We previously reported that other enzymes, such as phosphatases and lipases, are involved in mycoparasitism (Silva *et al.* 2009). Furthermore, using proteomic approaches, we recently identified a role for α-mannosidase and ABFase in mycoparasitism (Monteiro *et al.* 2010). In the present study, we observed that *T. asperellum* exhibited the highest CWDE activity. However, no species secreted a specific group of CWDEs. Moreover, we observed a high diversity of enzyme secretion, even within the same species. This finding can be explained by the high complexity of CWDE gene regulation, with different rates of evolution and adaptation, caused by different environmental pressures.

In the present study, we have revealed specific strains as strong candidates for the biological control of *S. sclerotiorum*. *Trichoderma asperellum* 364/01, *T. asperellum* 483/02, and *T. asperellum* 356/02 showed high and medium specific activities for key enzymes in the mycoparasitic process, e.g., chitinase, NAGase, β-1,3-glucanase, and proteases. Moreover, they demonstrated a high capacity to metabolize several substrates on Biolog FF Microplates (which is important for nutrient competition), and also a high acid phosphatase specific activity. Acid phosphatase is known to play a role in the

release of inorganic phosphate from the soil, thus making it available for use by plants (Altomare *et al.* 1999). Thus, the biological control of *S. sclerotiorum* by *T. asperellum* 364/01, *T. asperellum* 483/02, and *T. asperellum* 356/02 may also contribute to plant development.

Most CWDEs are inducible by substrate. Thus, the low specific activities of ABFase, α-mannosidase, and lipase exhibited by *Trichoderma* strains can be explained by the small amount of the respective substrates in the *S. sclerotiorum* cell wall (Bartnicki-Garcia 1968). Monteiro *et al.* (2010) reported high levels of α-mannosidase production by *T. harzianum* in the presence of *R. solani*, while Silva *et al.* (2009) observed the same pattern for lipase production by *Trichoderma reesei* during mycoparasitism against *Pythium ultimum*. Thus, it appears that secretion of these enzymes is phytopathogen-dependent.

High production of CWDEs does not guarantee successful biocontrol by *Trichoderma* in the field. The process is complex, and many other factors must be considered before choosing an isolate for field application. These include the ability of *Trichoderma* to produce and/or resist metabolites, antibiosis, rhizosphere modification, production of toxic compounds and/or antibiotics, plant growth promotion, stimulation of plant-defence mechanisms, and, most importantly, the survival of *Trichoderma* in the soil environment (reviewed by Benítez *et al.* 2004). Almeida *et al.* (2007) observed no correlation between CWDE production and frequency of coiling, during the screening of *Trichoderma* strains for biological control of *R. solani*. In the present study, we revealed a significant correlation ($r > 0.5$; $p < 0.01$) only between proteases, chitinase/antagonism, and glucanase/basic protease (data not shown). However, our outcomes should be treated cautiously, because of the small number of samples analyzed. Despite this limitation, the 21 isolates studied represent an important sample of the Brazilian diversity of *Trichoderma*, because they originated up to 1300 km apart, from latitudes ranging from 13°13′ to 23°25′. Thus, their diverse biochemical and metabolic profiles are likely to have been influenced by previous exposure to anthropogenic pressure in different agroecosystems. This selection pressure, in turn, may represent an advantage for conventional biological control.

In the present study, we revealed that antibiosis and antagonism against *S. sclerotiorum* were not species-dependent. Different *Trichoderma* strains of the same species exhibited different results, and no correlation between species was observed, despite a synergism between antibiosis and antagonism. Similar results were reported by Anees *et al.* (2010), during characterization of field isolates of *Trichoderma* for antagonism against *R. solani*. In the present study, we also detected no synergistic correlation between antagonism and CWDE production (data not shown), except for chitinase. *Trichoderma asperellum* 364/01, *T. asperellum* 483/02, and *T. asperellum* 356/02 exhibited high CWDE production, but a low capacity for antagonism. The cause of such a mismatch between CWDE production and antagonism/antibiosis remains to be elucidated. However, a *Trichoderma* strain with high expression of each trait would be desirable for biological control. Furthermore, metabolic capacity appeared not to be correlated with antagonism, because the best antagonists in the plate tests (ALL42, 11/11, and 29/11) showed different profiles in the Biolog FF Microplate analysis (blue rectangles on Fig 2). Taken together, these findings indicate the complexity of events that occur during biological control by *Trichoderma*.

In an attempt to develop a rapid screening system for biocontrol-competent strains of *Trichoderma*, Scherm *et al.* (2009) used subtraction hybridization to identify specific genes for use as markers. However, even in these groups of genes, the antagonistic activity appeared to be related to the efficiency of gene expression in the presence of the host, indicating diversity even within a species. These findings further confirm that the selection of candidates for biological control should be based primarily on the environmental fitness of competitive isolates and also on the type of pathogenic challenge rather than on the use of specific *Trichoderma* species. Thus, we propose that a combination of different *Trichoderma* species and strains, with different antagonistic and mycoparasitic characteristics, should be used for commercial biological control. Most importantly, strains should be previously tested for adaptation to the agroecosystem in which they will be applied. Thus, the development and use of a universal product for biological control requires extensive further research.

In the present study, we have successfully used molecular, enzymatic, and metabolic analyses to identify potential *Trichoderma* strains for use in the biological control of *S. sclerotiorum*. We have further demonstrated that CWDEs represent important tools for the identification of promising candidates for biological control. Our data, when combined with the results of field testing (currently underway), will be crucial to the selection of *Trichoderma* strains for more efficient formulations for the control of fungal pathogens.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.funbio.2012.04.015.

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